Up-regulation of Expression of the Ubiquitin Carboxyl-Terminal Hydrolase L1 Gene in Human Airway Epithelium of Cigarette Smokers

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Abstract

Neuroendocrine differentiation is a common feature of lung cancer and increased numbers of neuroendocrine cells and their peptides have been described in chronic smokers. To understand the effects of cigarette smoking on the gene expression profile of neuroendocrine cells, microarray analysis with TaqMan confirmation was used to assess airway epithelial samples obtained by fiberoptic bronchoscopy from 81 individuals [normal nonsmokers, normal smokers, smokers with early chronic obstructive lung disease (COPD), and smokers with established COPD]. Of 11 genes considered to be neuroendocrine cell specific, only ubiquitin carboxyl-terminal hydrolase L1 (UCHL1), a member of the ubiquitin proteasome pathway, was consistently up-regulated in smokers compared with nonsmokers. Up-regulation of UCHL1 at the protein level was observed with immunohistochemical analysis of bronchial biopsies of smokers compared with nonsmokers. UCHL1 expression was evident only in neuroendocrine cells of the airway epithelium in nonsmokers; however, UCHL1 was also expressed in ciliated epithelial cells in smokers. This observation may add further weight to recent observations that ciliated cells are capable of transdifferentiating to other airway epithelial cells. In the context that UCHL1 is involved in the degradation of unwanted, misfolded, or damaged proteins within the cell, and is overexpressed in >50% of lung cancers, its overexpression in chronic smokers may represent an early event in the complex transformation from normal epithelium to overt malignancy. (Cancer Res 2006; 66(22): 10729-40)

Introduction

Neuroendocrine cells, flask-like cells present in sparse numbers in the airway epithelium, are believed to play an important role in lung physiology with regard to vascular control, inflammation, and responses to hypoxia by virtue of their ability to produce and secrete a variety of active peptides (1–3). In the healthy adult human lung, neuroendocrine cells are rare, with estimated numbers of 1 per 2,500 epithelial cells, mostly concentrated in the intrapulmonary airways (4). The majority of airway neuroendocrine cells exist as solitary cells but some are present in clusters termed neuroepithelial bodies (1–3). Neuroendocrine cells are relevant to lung cancer in that ~100% of small cell and 10% to 15% of non–small cell lung cancers have neuroendocrine features (5–7). Immunohistologic assessment of neuroendocrine-specific gene products is routinely used to assess lung tumors, and for non–small cell lung cancer, neuroendocrine features are proposed to predict responses to chemotherapy and overall survival (8–11). One such neuroendocrine cell-specific product is ubiquitin carboxyl-terminal hydrolase L1 (UCHL1; also called protein gene product 9.5), a 24,000 Da peptide normally expressed in neurons and cells of the neuroendocrine system (1–3, 12, 13). UCHL1 is a member of the ubiquitin proteasome pathway controlling intracellular protein degradation, functioning to maintain ubiquitin balance by associating with ubiquitin and by releasing ubiquitin from tandemly conjugated ubiquitin monomers and small adducts or unfolded proteins (14). Other functions ascribed to UCHL1 include ubiquitin ligase activity and stabilization of ubiquitin within the cell, and it may serve as a regulator of apoptosis (15–19). Immunohistologic assessment of lung tumors shows that >50% of non–small cell lung cancers express UCHL1, correlating with a more advanced cancer stage (20, 21).

Based on the knowledge that cigarette smoking is the leading cause of lung cancer (22), increased numbers of neuroendocrine cells are present in the airways of cigarette smokers with lung disease (23), increased levels of neuroendocrine peptides like bombesin and calcitonin are found in biological fluids of healthy smokers (24–26), and that the UCHL1 protein is used as a marker of lung cancer (20), we asked: Does cigarette smoking up-regulate the expression of the UCHL1 gene in the normal (i.e., nonmalignant) airway epithelium? To evaluate this question, large and small airway epithelium obtained by fiberoptic bronchoscopy and brushing from 81 individuals [phenotypically normal nonsmokers and normal smokers, as well as individuals with early and established chronic obstructive lung disease (COPD)] were assessed for the expression of 11 neuroendocrine cell–specific genes using Affymetrix microarrays with TaqMan RT-PCR confirmation. We found that the expression of neuroendocrine cell–specific genes was not significantly altered by cigarette smoking except for a consistent up-regulation of expression of UCHL1 in almost all smokers. Immunofluorescence staining of bronchial biopsies showed that, in addition to being present in neuroendocrine cells of nonsmokers and smokers, UCHL1 was expressed in ciliated epithelial cells in smokers. In light of recent observations in experimental animals that airway ciliated cells can transdifferentiate to nonciliated cells (27, 28), and in the context that UCHL1 is overexpressed in many lung cancers (20), its up-regulation in response to cigarette smoking may represent one of the early events in the complex progression from normal epithelium to neoplastic transformation.
**Materials and Methods**

**Study population.** Normal nonsmokers, healthy chronic smokers, smokers with early COPD, and smokers with established COPD were evaluated at the Weill Cornell NIH General Clinical Research Center under protocols approved by the Weill Cornell Medical College Institutional Review Board [different arrays were used to assess the total of 114 samples from 81 individuals; the demographic data for each group and for each site (large and small airway epithelium) are presented in Table 1]. Written informed consent was obtained from each individual before enrollment in the study. No individual in any study group had any variable that suggested evidence of a lung malignancy. Normal nonsmokers and normal smokers were determined to be phenotypically normal on the basis of clinical history and physical examination, routine blood screening tests, urinalysis, chest X-ray, electrocardiogram, and pulmonary function testing. Current smoking status was confirmed on history, venous carboxyhemoglobin levels, and urinalysis for nicotine levels and its derivative cotinine. Smokers were defined as having early COPD if they had a diffusion capacity for carbon monoxide (DLCO) of <80% predicted with no evidence of airflow obstruction on pulmonary function testing and/or high-resolution computed tomography scanning of the chest revealed evidence of emphysema. Smokers with established COPD were defined according to Global Initiative for Chronic Obstructive Lung Disease criteria (29).

**Sampling of airway epithelial cells.** Epithelial cells from the large and small airways were sampled using fiberoptic bronchoscopy as previously described (30, 31). Smokers were asked not to smoke the evening before the procedure. After achieving mild sedation and anesthesia of the vocal cords, a fiberoptic bronchoscope (Pentax, EB-1530T3) was advanced to the desired bronchus. Large airway epithelial samples were collected by gentle brushing of the third- to fourth-order bronchi and small airway samples were collected from 10th- to 12th-order bronchi. These cells were subsequently collected in 5 mL of bronchial epithelium basal cell medium (Clonetics, Walkersville, MD). An aliquot was used for cytology and differential cell count and the remainder was processed immediately for RNA extraction. Total cell counts were obtained using a hemocytometer, whereas differential cell counts (epithelial versus inflammatory) were determined on sedimented cells prepared by centrifugation (Cytospin11, Shandon Instruments, Pittsburgh, PA) and stained with DiffQuik (Baxter Healthcare, Miami, FL).

**RNA extraction and microarray processing.** Analyses were done using three different Affymetrix (Santa Clara, CA) microarrays, including HuGeneFL array (7,000 probe sets), HG-U133A array (22,000 probe sets), and HG-U133 Plus 2.0 array (54,000 probe sets). The protocols used were as described by the manufacturer. Total RNA was extracted from epithelial cells using TRIzol (Invitrogen, Carlsbad, CA) followed by RNeasy (Qiagen, Valencia, CA) to remove residual DNA. This process yielded 2 to 4 μg RNA per 10⁶ cells. Samples analyzed using the HuGeneFL and HG-U133A microarrays were processed as previously described (30, 31), using 6 μg RNA. For samples analyzed using the HG-U133 Plus 2.0 array, an aliquot of each RNA sample was run on an Agilent Bioanalyzer (Agilent Technologies, Palo Alto, CA) to visualize and quantify the degree of RNA integrity. The concentration was determined using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). Three quality control criteria were used for an RNA sample to be accepted for further processing: (a) A₂₆₀/A₂₃₀ ratio between 1.7 and 2.3; (b) concentration within the range of 0.2 to 6 μg/mL; and (c) Agilent electropherogram displaying two distinct peaks.
A list of known neuroendocrine cell–specific genes was established from smokers, smokers with early COPD, and smokers with established COPD, expression in the large and small airway samples of nonsmokers, healthy nonsmokers, and smokers. The Affymetrix U133A data and HG-U133 Plus 2.0 large airway data was log transformed expression level for all the genes across all arrays in a data set. All HG-U133A and HG-U133 Plus 2.0 arrays were hybridized to test chips with a $3^\Delta C_t$ ratio of <3 were deemed satisfactory.

To maintain quality, only samples hybridized to test chips with a $3^\Delta C_t$ ratio of <3 were deemed satisfactory.

**Table 1. Study population and airway epithelial samples (Cont’d)**

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peaks corresponding to the 28S and 18S rRNA bands at a ratio of 28S/18S of >0.5 with minimal or no degradation. Double-stranded cDNA was synthesized from 3 μg of total RNA using the GeneChip One-Cycle cDNA Synthesis kit, followed by cleanup with GeneChip Sample Cleanup Module, in vitro transcription (IVT) reaction using the GeneChip IVT Labeling kit, and clean-up and quantification of the biotin-labeled cDNA yield by spectrophotometric analysis. All kits were from Affymetrix. Hybridizations to test chips and to the microarrays were done according to Affymetrix protocols, and microarrays were processed by the Affymetrix fluids station and scanned with an Affymetrix GeneArray 2500 (HuGeneFL) and the Affymetrix GeneChip Scanner 3000 7G (HG-U133A and HG-U133 Plus 2.0). To maintain quality, only samples hybridized to test chips with a 3' to 5' ratio of <3 were deemed satisfactory.

**Microarray data analysis.** Captured images were analyzed using Microarray Suite version 5.0 algorithm (Affymetrix). These data were normalized using GeneSpring version 6.2 software (Agilent Technologies) as follows: (a) per array, by dividing raw data by the 50th percentile of all measurements; and (b) per gene, by dividing the raw data by the median expression level for all the genes across all arrays in a data set. All HG-U133A and HG-U133 Plus 2.0 large airway data was log transformed before statistical analysis. To evaluate neuroendocrine cell–specific gene expression in the large and small airway samples of nonsmokers, healthy smokers, smokers with early COPD, and smokers established COPD, a list of known neuroendocrine cell–specific genes was established from the literature (1–3, 32–34). This signature transcriptome of neuroendocrine cells was used to assess the effects of smoking on the genome of these cells. Expression was defined as having an Affymetrix Detection Call of Present (P call) in ≥50% of samples assessed by each type of microarray.

**TaqMan reverse transcription-PCR confirmation of microarray expression levels.** TaqMan real-time reverse transcription-PCR (RT-PCR) was done on available RNA samples from the small airways of 12 normal nonsmokers and 10 normal smokers that had been assessed with the HG-U133 Plus 2.0 array. cDNA was synthesized from 2 μg RNA in a 100 μl reaction volume, using the TaqMan Reverse Transcriptase Reaction kit (Applied Biosystems, Foster City, CA), with random hexamers as primers. Two dilutions of 1:50 and 1:100 were made from each sample and triplicate wells were run for each dilution. TaqMan PCR reactions were carried out using premade gene expression assays for neuroendocrine genes from Applied Biosystems and 2 μl cDNA were used in each 25-μl reaction volume. The endogenous control was 18S rRNA and relative expression levels were determined using the DD$C_t$ method (Applied Biosystems) with the average value for the nonsmokers as the calibrator. The PCR reactions were run in an Applied Biosystems Sequence Detection System 7500.

**Localization of UCHL1 in the airway epithelium.** To determine which airway epithelial cells express UCHL1, bronchial biopsies were obtained from the large airway epithelium of six nonsmokers and six normal smokers using conventional methods. Immunohistochemistry was subsequently done on paraffin-embedded endobronchial biopsies. Sections were deparaffinized and rehydrated through a series of xylene and alcohol. To enhance staining, an antigen retrieval step was carried out by microwave treatment of the sections at 100°C for 15 minutes in citrate buffer solution (Labvision, Fremont, CA) followed by cooling at 23°C for 20 minutes. Endogenous peroxidase activity was quenched using 0.3% H2O2 and blocking with normal goat serum to reduce background staining. Samples were incubated with the primary antibody at 23°C for 1 hour. For chromogranin A (CHGA), the primary antibody was mouse monoclonal (LK2H10 + PHE5) anti-human antibody (Labvision) diluted 1:5000 and rabbit IgG (DakoCytomation, Carpinteria CA) was the isotype control. For UCHL1 detection, the primary antibody was rabbit polyclonal anti-human UCHL1 (Labvision) diluted 1:2500 and rabbit IgG (DakoCytomation, Carpinteria CA) was the isotype control.
control. To block UCHL1 antibody binding, the UCHL1 antibody was incubated with the full-length recombinant UCHL1 protein (Labvision) at 23°C for 30 minutes to saturate binding sites before being applied to sample tissues. Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA) and 3, 3-diaminobenzidine substrate kit (Vector Laboratories) were used to visualize antibody binding. The sections were counterstained with hematoxylin (Sigma Aldrich, St. Louis, MO) and mounted using GVA mounting medium (Zymed, San Francisco, CA). Brightfield microscopy was done using a Nikon Microphot microscope equipped with a Plan Neofluor objective lens. Images were captured with an Olympus DP70 CCD camera.

Immunofluorescent staining was carried out on airway epithelial biopsies using primary antibodies for UCHL1 and CHGA as described above; mouse monoclonal (ONS1A6) anti-human β IV tubulin (1/500 dilution; Biogenex, San Ramon, CA) as a marker for ciliated cells (35); mouse monoclonal (45M1) mucin 5AC (1/200; Labvision) as a marker for secretory cells (36); and mouse monoclonal (SH-L1) S100 A2 (1/50 dilution; Biogenex, San Ramon, CA) as a marker for basal cells (37). Following incubation with the primary antibodies at 23°C for 1 hour in a humidified chamber, goat anti-rabbit Cy5 conjugated AffiniPure F(ab)2 (Jackson Immunoresearch, West Grove, PA) at 1/100 dilution was used as a secondary antibody for UCHL1 and goat anti-mouse Cy3-conjugated AffiniPure F(ab)2 (Jackson Immunoresearch) at 1/100 dilution was used as a secondary antibody for all other antibodies. Fluorescence microscopy was done using a Zeiss LSM 510 Laser Scanning Confocal microscope equipped with a Plan Neofluor ×40 NA 0.75 objective lens. Illumination was provided by an argon laser (488 nm line) and two helium/neon lasers (543 and 633 nm lines) with matched dichroic mirrors and emission filters. Images were analyzed using Zeiss LSM Image Browser version 3.1.0.99. Pseudocolor images were formed by encoding Cy5 fluorescence in the green channel, Cy3 fluorescence in the red channel, and autofluorescence in gray scale. The images were composed by integrating five independent images collected at a step size of 1.7 μm.

**Statistical analysis.** For all HuGeneFL data and small airway data analyzed on the HG-U133 Plus 2.0, P values for all comparisons were calculated using a two-tailed t test, assuming unequal variance (Welch t test) with the Benjamini-Hochberg multiple test correction for false-discovery rate (38), using GeneSpring software. Genes were considered significant if the Benjamini-Hochberg corrected P value was <0.05. For HG-U133A large and small airway data and for large airway data analyzed on the HG-U133 Plus 2.0 microarray, P values were calculated as described above, but in the absence of the Benjamini-Hochberg correction. Average expression values for neuroendocrine cell–specific genes in large and small airway samples were calculated from normalized expression levels for nonsmokers, normal smokers, smokers with early COPD, and smokers with established COPD. TaqMan data was normalized per gene by dividing by the median expression of each gene in all samples, and subsequently the mean and SE were calculated for normalized values of expression. P values for TaqMan data were calculated using the Welch t test.

**Web deposition of data.** All data has been deposited in the Gene Expression Omnibus site, which is curated by the National Center for Biotechnology. Accession numbers include (a) HuGeneFL accession number GSE5056; (b) small airways HG-U133A, accession number GSE3320, already cited in ref. 31; (c) large Airways HG-U133A accession number GSE5057; (d) large airways HG-U133 Plus 2.0 accession number GSE5059; and (e) small airways HG-U133 Plus 2.0 accession number GSE5058 (31).

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**Table 2. Microarray assessment of neuroendocrine cell–specific genes in normal nonsmokers, normal smokers, smokers with early COPD, and smokers with established COPD**

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>HUGene FL chip</th>
<th>%P call</th>
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<tbody>
<tr>
<td></td>
<td>Large airways</td>
<td>Normal nonsmokers</td>
</tr>
<tr>
<td></td>
<td>Small airways</td>
<td>Normal nonsmokers</td>
</tr>
<tr>
<td></td>
<td>Large airways</td>
<td>Normal nonsmokers</td>
</tr>
</tbody>
</table>

Abbreviation: NA, probe not on array, not applicable.

*Overall assessment of expression was based on P call ≥50% in at least two of the three arrays used. ‡ means probably expressed, but observed in only one type of array; this may be dependent on different probes on the different arrays.

*Early COPD smokers—smokers with normal lung function except for abnormal DLCO.

§Established COPD smokers—smokers with COPD as defined by the GOLD criteria (29).

>“No” indicates not expressed with a P call <50% and “Yes” indicates expression with a P call ≥50% (bold type).

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Results

Study population. A total of 114 samples were assessed from 81 study individuals (Table 1). Results were obtained using three different microarrays including: (a) HuGeneFL microarray—18 large airway samples from 9 nonsmokers and 26 large airway samples from 13 normal smokers; (b) HG-U133A array—large and small airway samples from 5 nonsmokers and 6 normal smokers; and (c) HG-U133 Plus 2.0 array—large airway samples from 4 normal smokers, 5 normal smokers, and small airway samples from 12 normal nonsmokers, 12 normal smokers, 9 smokers with early COPD, and 6 smokers with established COPD. All individuals had no significant prior medical history and normal physical examinations. There were no differences between groups with regard to sex, race, or age (P > 0.05 for all comparisons). There was a statistically significant difference in age in the nonsmoker group versus early COPD group (P < 0.01) analyzed with the HG-U133 Plus 2.0 microarray. All individuals were HIV negative with blood and urine variables within reference ranges (P > 0.05 all comparisons). Smokers had an average smoking history of 27 ± 2 pack-years. The number of cells recovered by brushing ranged from 4.9 × 10^6 to 9.8 × 10^6 (Table 1). In all cases, >95% of cells recovered were epithelial cells. The subtypes of airway epithelial cells were as expected from the large and small airways (Table 1; ref. 29). Neuroendocrine cells were not observed in brushed airway samples.

Detection of neuroendocrine gene expression in the large and small airway epithelium. With the criteria of P call ≥ 50%, most neuroendocrine genes were not detected in the large airway epithelium of nonsmokers [secretory granule neuroendocrine peptide 1 (SGNE1), pro-enkephalin (PENK), tachykinin 1 (TAC1), achaete scute homologue 1 (ASCL1), neuronal cell adhesion molecule 1 (NCAM1), calcitonin gene-related polypeptide β (CALCB), CHGA, gastrin releasing peptide (GRP), and UCHL1 (Table 2)]. Of the 11 neuroendocrine genes evaluated, only expression of enolase 2 (ENO2) was universally detected in the large airways of nonsmokers. In the small airways of normal nonsmokers, 5 of the 11 neuroendocrine genes were not expressed (SGNE1, PENK, TAC1, ASCL1, CALCB, and UCHL1), one gene was equivocal (NCAM1 was detected in only the HG-U133 Plus 2.0 array), and four were clearly detected [secretogranin 2 (SGCG2), CHGA, EN02, and GRP].

In the current smokers (phenotypically normal, early COPD, and established COPD), expression of the neuroendocrine-specific genes in the large and small airways was mostly consistent with that observed in the large and small airway epithelium in nonsmokers (Table 2). However, in marked contrast to the other neuroendocrine genes, whereas UCHL1 was not detected in any of the large and small airway epithelial samples of the nonsmokers, UCHL1 was detected in the large and small airway epithelium of smokers in almost every microarray (large airway epithelium samples – 69% of normal smokers assessed with the HuGeneFL chip; 100% of normal smokers with HG-U133A and 80% of normal smokers with HG-U133 Plus 2.0; small airway epithelium samples – 100% of normal smokers assessed with HG-U133A; 100% of normal smokers with HG-U133 Plus 2.0; and 89% early COPD smokers with HG-U133 Plus 2.0, and 100% of established COPD smokers with HG-U133 Plus 2.0).

UCHL1 expression was 18.3-fold higher in normal smokers compared with nonsmokers in the large airways analyzed with the HuGeneFL array (P < 0.01), 9.0-fold higher in large airways analyzed with the HG-U133A array (P < 0.01), and 42.2-fold higher in large airways analyzed with the HG-U133 Plus 2.0 array (P < 0.01). In the small airways, UCHL1 was 11.4-fold higher in normal smokers than nonsmokers in HG-U133A array (P < 0.01). In the HG-U133 Plus 2.0 data set, UCHL1 expression was 39.3-fold higher in normal smokers (P < 0.01), 60.8-fold higher in smokers with early COPD (P < 0.01), and 38.6-fold higher in smokers with established COPD (P < 0.01). There was no significant difference in the level of expression of UCHL1 between normal smokers and smokers with early COPD (P > 0.8) or smokers with established COPD (P > 0.9; Fig. 1).

Quantitative expression of the neuroendocrine cell–specific genes in the small airway epithelium. Of the neuroendocrine-specific genes expressed in the airway epithelium, quantitative

<table>
<thead>
<tr>
<th>Table 2. Microarray assessment of neuroendocrine cell–specific genes in normal nonsmokers, normal smokers, smokers with early COPD, and smokers with established COPD (Cont’d)</th>
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%P call

<ref>Figure 1</ref>
assessment of the relative gene expression levels showed no difference among nonsmokers and smokers for GRP, ENO2, or SCG2 (Fig. 2; \(P > 0.1\) for all comparisons of nonsmokers to each of the current smoker groups including phenotypically normal smokers, smokers with early COPD, and smokers with established COPD). There was a significant difference in expression levels of CHGA in smokers with established COPD compared with normal nonsmokers (\(P < 0.04\)).

In marked contrast, the expression of UCHL1 was up-regulated in smokers compared with nonsmokers in the small and large airway epithelium in all the data sets (Fig. 1). This was true for normal smokers compared with normal nonsmokers in the large airway epithelial samples assessed with the HuGeneFL microarray (\(A; P < 0.01\)); normal smokers compared with normal nonsmokers of the large airway epithelium assessed with the HG-U133A array (\(B; P < 0.01\)); normal smokers compared with normal nonsmokers of the small airway epithelium assessed with the HG-U133 Plus 2.0 array (\(C; P < 0.01\)); and normal smokers compared with normal nonsmokers of the small airway epithelium with the HG-U133 Plus 2.0 array (\(C\)).

TaqMan RT-PCR confirmation of microarray results. To confirm the results obtained from microarray studies, TaqMan RT-PCR was carried out on RNA samples from the small airways of 12 normal nonsmokers and 10 normal smokers (Fig. 3). The TaqMan data confirmed that there was no difference in expression levels of UCHL1 in smokers compared with nonsmokers for GRP, ENO2, or SCG2 (Fig. 2; \(P > 0.1\) for all comparisons of nonsmokers to each of the current smoker groups including phenotypically normal smokers, smokers with early COPD, and smokers with established COPD). There was a significant difference in expression levels of CHGA in smokers with established COPD compared with normal nonsmokers (\(P < 0.04\)).

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levels of other neuroendocrine-specific genes, including CHGA, GRP, EN02, and SCG2. The TaqMan analysis also confirmed the up-regulation of UCHL1 mRNA expression in normal smokers compared with nonsmokers \((P < 0.01)\).

**Localization of UCHL1 in the airway epithelium of smokers.** Immunohistochemistry was used to assess expression of CHGA and UCHL1 in endobronchial biopsies obtained from large airways at bronchoscopy from six nonsmokers and six normal smokers. This analysis showed protein expression of CHGA and UCHL1 in airway epithelial cells with the typical morphology and localization of neuroendocrine cells (Fig. 4A-C). Surprisingly, the smokers not only had UCHL1 expression in typical neuroendocrine cells, but there was also positive staining for UCHL1 in other epithelial cells more apically in the airway epithelium that was not present in nonsmokers (Fig. 1E, F). To confirm the specificity of the polyclonal rabbit anti-UCHL1 antibody, a blocking step was done with full-length recombinant UCHL1 protein; this completely blocked all antibody binding on biopsy samples from smokers, demonstrating the specificity of this polyclonal antibody for the UCHL1 epitope (not shown). Overall, although there were a greater number of cells with a neuroendocrine morphology observed in the airway epithelium of normal smokers compared with nonsmokers, there were also a greater number of UCHL1-positive cells within the airway epithelium of smokers compared with nonsmokers that were not positive for the neuroendocrine marker CHGA. These additional UCHL1-positive cells had the appearance and morphology of ciliated epithelial cells (Figs. 5 and 6). UCHL1 was confirmed to be present in ciliated airway epithelial cells in the smokers as evidenced by colocalization with the ciliated cell–specific marker \(\beta IV\) tubulin but not with the secretory cell marker MUC5AC. The colocalization of UCHL1 and \(\beta IV\) tubulin was almost universal throughout the cilia with some cilia being more intensely positive for UCHL1, whereas, as expected, all cilia stained positive for \(\beta IV\) tubulin. UCHL1 was not present in basal cells as evidenced by lack of colocalization with S100 A2, a marker of these cells (not shown).

**Discussion**

Cigarette smoking is the major risk factor associated with the development of lung cancer (22). In the present study, we show that UCHL1, a ubiquitin thiolesterase and member of the ubiquitin

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**Figure 2.** Normalized expression levels of the neuroendocrine cell–specific genes ASCL1, SCG2, CHGA, EN02, and GRP in human small airway epithelium assessed with the HG-U133 Plus 2.0 array in 12 normal nonsmokers, 12 normal smokers, 9 individuals with early emphysema, and 6 individuals with established COPD. X axis, the neuroendocrine-specific gene; Y axis, normalized gene expression levels. Each symbol represents an individual: nonsmokers (△), normal smokers (▲), early COPD (○), and individuals with established COPD (◆).
proteasome pathway, is consistently up-regulated in the large and small airway epithelium of cigarette smokers, including normal smokers, smokers with early COPD, and smokers with established COPD. These observations are relevant to lung cancer in several respects. First, UCHL1 is commonly used as a marker in assessing lung cancer, with 100% of small cell cancers and 50% to 60% of non–small cell cancers expressing UCHL1. The expression of UCHL1 has also been linked to outcome (20, 21). Second, the up-regulation of an enzyme linked to the degradation of misfolded proteins is relevant to the multifactorial, complex pathogenesis of lung cancer. Third, the observation that much of the up-regulated UCHL1 in the smoker airway epithelium was not only in neuroendocrine cells, but also in ciliated cells, together with the observation that ciliated cells can trans-differentiate to other cell types (27, 28), leads to the provocative question as to whether some lung cancers are derived from cells that were originally differentiated ciliated cells.

**Neuroendocrine-specific gene expression in the airway epithelium.** Using Affymetrix microarrays with TaqMan confirmation, assessment of gene expression of neuroendocrine-relevant genes in the large and small airway epithelium of healthy nonsmokers, phenotypically normal smokers, smokers with early COPD, and smokers with established COPD showed that smoking did not alter the expression of genes coding for most neuroendocrine peptides at the mRNA level, but there was consistent up-regulation of UCHL1 in smokers.

Consistent with the sparse numbers of neuroendocrine cells present in the airway epithelium (0.3-0.5% of airway epithelial cells; ref. 39), expression of many neuroendocrine cell–specific genes could not be detected in the brushed samples of large and small airway epithelium. Using the criteria of an Affymetrix Detection Call of Present in ≥50% samples, the neuroendocrine genes that were expressed (other than UCHL1 in smokers) included GRP, CHGA, neuron-specific enolase, and SGC2. Although previous studies have described increased numbers of neuroendocrine cells in the airways and their peptides in biological fluids of smokers with a variety of pulmonary disease states (23, 40), the data did not show increased expression of the mRNA for these peptides, suggesting that smoking may induce the release of some neuroendocrine peptides rather than up-regulating the expression of most of the neuroendocrine cell–relevant genes (24–26).

**UCHL1 gene expression in the airway epithelium.** The observation that UCHL1 is up-regulated in smokers compared with nonsmokers is important in the context that ubiquitination of proteins is an important control process within cells, targeting them for localization and degradation, particularly oxidized or misfolded proteins (41, 42). Much of the knowledge of the function of UCHL1 is from the gracile axonal dystrophy mouse, a naturally occurring mutant that lacks the UCHL1 protein due to a deletion of UCHL1 in the smoker airway epithelium was not only in neuronal cells that are composed of ubiquitin and abnormal proteins (44). Under normal conditions, when proteins are tagged with ubiquitin, they are targeted for degradation within the cell. UCHL1 acts to maintain ubiquitin levels within the cell by its physical association with ubiquitin, by hydrolyzing bonds to release monoubiquitin from polyubiquitin chains and cleaving bonds between ubiquitin and small adducts before degradation by the proteasome (14). UCHL1 also plays a role in programmed cell death through its posttranslational regulation of apoptotic factors (15–17, 19). In this regard, there is reduced apoptosis in the testes of UCHL1-deficient mice under cryptorchid-induced stress and in the retina under ischemic stress (15–17). This is associated with an alteration in the balance of proapoptotic and antiapoptotic factors, particularly of the bcl-2 family (15–17). In the context that UCHL1 plays a role in apoptosis by altering the balance of apoptotic factors, its up-regulation in smokers may reflect increased cell stress, with cigarette smoke altering protein structures within the cell. A hereditary form of Parkinson’s disease has also been linked to an I93M mutation in the UCHL1 gene (45), consistent with a proposed role of UCHL1 in maintaining the balance of normal proteins within the cell (46, 47).

UCHL1 may be a marker of cellular stress in lung cancer. The ubiquitin proteasome pathway regulates with exquisite specificity cellular processes, such as cell cycle progression, inhibition, or execution of apoptosis and activation or expression of transcription factors, including p53, c-Jun, and HIF1 (35, 48, 49). UCHL1 has been shown to interact with JAB1, a Jun activation domain binding protein that can bind to p27Kip1, a cyclin-dependent protein kinase inhibitor involved in cell cycle regulation (35). UCHL1 associates with JAB1 in lung cancer cell lines and its overexpression has been linked to decreased levels of p27Kip1, which is observed in many lung cancers (35). Immunohistochemistry for UCHL1 is frequently used in the evaluation of small cell and non–small cell lung cancer (20, 21). UCHL1 is highly expressed in >50% of primary lung cancers, most lung cancer cell lines, and some nonpulmonary cancers like pancreatic and esophageal cancer (50). The level of expression in lung cancer increases with increasing tumor stage and this expression may occur independently of overt neuroendocrine differentiation (20).

Although the data in the present study is insufficient to determine the temporal role, if any, of UCHL1 in the progression of smoking-induced neoplastic transformation, the data set of Spira et al. (GSE994) of airway epithelium gene expression includes a group of ex-smokers who had baseline levels of UCHL1.

![Figure 3. Confirmation of microarray results with TaqMan real-time RT-PCR for five neuroendocrine cell–specific genes, including CHGA, GRP, ENO2, SGC2, and UCHL1. The data include samples from the small airways from 12 nonsmokers and 10 smokers. X axis, neuroendocrine-specific genes evaluated; Columns, average expression levels on a logarithmic scale. Expression levels are normalized by dividing individual values by the median expression level for each gene in all nonsmokers and smokers. Bars, SE. P values represent the comparison between nonsmokers and smokers.](http://www.aacrjournals.org/doi/abs/10.1158/0008-5472.CAN-06-3567)
This suggests that expression of UCHL1 may return to the level of never smokers upon smoking cessation, at least in some people.

It is observed in this study that many of the UCHL1-positive cells in the airway epithelium of smokers are not positive for other neuroendocrine peptides like CHGA, and that many of the UCHL1-positive cells have the morphology of ciliated epithelial cells. Immunofluorescent staining and confocal microscopy confirmed the presence of UCHL1 in ciliated epithelial cells where it was colocalized with β IV tubulin. UCHL1 expression in ciliated epithelial cells was only present in smokers, whereas it was expressed in neuroendocrine cells in both nonsmokers and smokers, where it was colocalized with CHGA. UCHL1 was not present in other epithelial cells like secretory cells and basal cells, as it did not colocalize with MUC5AC or S100 A2, markers for these cells, respectively. Ciliated epithelial cells have traditionally been thought of as terminally differentiated cells, incapable of further change, but recent studies point to the plasticity of these cells and the role they have to play in the repair process of the respiratory epithelium (27, 28). The observation that UCHL1 is up-regulated in ciliated epithelial cells in cigarette smokers is important because UCHL1 is traditionally a marker of neuroendocrine cells and a commonly used marker for lung cancer.

In summary, UCHL1, a gene that is overexpressed in many lung cancers, is expressed in neuroendocrine cells of nonsmokers and in neuroendocrine and ciliated epithelial cells of smokers. This may represent a very early event in the complex processes related to the repair and differentiation of the airway epithelium.
changes that occur in the spectrum from normal cells to overt malignancy. Further studies are required to advance the understanding of the role of UCHL1 in epithelial cell function and in the pathogenesis of pulmonary disease and cancer.

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Figure 5. Confocal immunofluorescent assessment of UCHL1 expression in the airway epithelium of normal smokers. A, negative controls, rabbit IgG isotype control (green), mouse IgG1 isotype control (red), and autofluorescence (gray). UCHL1 colocalizes with CHGA to neuroendocrine cells (arrows), but is also expressed in nonneuroendocrine cells (open arrowheads). B, normal smoker, anti-UCHL1 antibody (green), anti-CHGA antibody (red), and autofluorescence (gray). UCHL1 colocalizes with CHGA to neuroendocrine cells (arrows), but is also expressed in nonneuroendocrine cells (open arrowheads). C, normal smoker, anti-MUC5AC antibody, and anti-UCHL1 antibody; anti-MUC5AC antibody (red), anti-UCHL1 antibody (green). Mucin containing secretory cells (closed arrows), UCHL1-positive airway epithelial cells (closed arrowheads), and nerves (open arrow). D, normal smoker, anti-βIV tubulin antibody, and anti-UCHL1 antibody; anti-βIV tubulin antibody (red) and anti-UCHL1 antibody (green), colocalization of β IV tubulin and UCHL1 (yellow-orange, closed arrowheads). All panels, bar 20 μm.

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Up-regulation of Expression of the \textit{Ubiquitin Carboxyl-Terminal Hydrolase L1} Gene in Human Airway Epithelium of Cigarette Smokers

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